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A poly(D,L-lactide) resin for the preparation of tissue engineering scaffolds by stereolithography

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Abstract

Porous polylactide constructs were prepared by stereolithography, for the first time without the use of reactive diluents. Star-shaped poly(D,L-lactide) oligomers with 2, 3 and 6 arms were synthesised, end-functionalised with methacryloyl chloride and photo-crosslinked in the presence of ethyl lactate as a non-reactive diluent. The molecular weights of the arms of the macromers were 0.2, 0.6, 1.1 and 5 kg/mol, allowing variation of the crosslink density of the resulting networks. Networks prepared from macromers of which the molecular weight per arm was 0.6 kg/mol or higher had good mechanical properties, similar to linear high molecular weight poly(D,L-lactide). A resin based on a 2-armed poly(D,L-lactide) macromer with a molecular weight of 0.6 kg/mol per arm (75 wt%), ethyl lactate (19 wt%), photo-initiator (6 wt%), inhibitor and dye was prepared. Using this resin, films and computer-designed porous constructs were accurately fabricated by stereolithography. Pre-osteoblasts showed good adherence to these photo-crosslinked networks. The proliferation rate on these materials was comparable to that on high molecular weight poly(D,L-lactide) and tissue culture polystyrene.

Introduction

Tissue engineering scaffolds prepared by rapid prototyping techniques have several advantages when compared to those fabricated by conventional techniques such as porogen leaching, phase-separation/freeze-drying and gas-foaming. These conventional techniques often result in inhomogeneous scaffolds with broad pore size distributions, poor pore interconnectivity and inferior mechanical properties. Rapid prototyping allows computer-designed architectures to be built in a reproducible manner. This enables the preparation of scaffolds with optimal properties regarding pore structure and connectivity, geometry, mechanical properties, cell-seeding efficiency, and transport of nutrients and metabolites [1]. Of the rapid prototyping techniques, stereolithography is the most versatile method with the highest accuracy and precision [2]. Its working principle is based on spatially controlled solidification of a liquid photo-polymerisable resin. Using a computer-controlled laser beam or a digital light projector with a computer-driven building stage, a solid, 3-dimensional object can be constructed in a layer-by-layer fashion.

The number of stereolithography resins available for use in biomedical applications is limited. Poly(ethylene glycol)dimethacrylate has been used to fabricate non-degradable cell-containing hydrogels in pre-designed shapes [3-5]. In tissue engineering however, resorbable constructs are desired. The only biodegradable macromers that have been applied are based on trimethylene carbonate and ϵ -caprolactone oligomers [6, 7], or on poly(propylene fumarate). The latter requires a reactive diluent such as diethyl fumarate to obtain an appropriate reaction rate and viscosity of the resin [8]. Upon photo-polymerisation of these resins, networks are formed with low glass transition

temperatures and low E-modulus values under physiological conditions. For tissue engineering of hard tissues such as bone, strong and rigid biodegradable materials are desired. Polylactide is such a material, and it has a long track record of successful application in the clinic and in the preparation of tissue engineering scaffolds. The amorphous form, poly(D,L-lactide) (PDLLA), has successfully been applied in resorbable bone fixation devices clinically [9, 10] and for scaffolds that proved well-suited for bone tissue engineering [11-13]. PDLLA has a glass transition temperature of approximately 55 °C, and an elasticity modulus close to 3 GPa; it is one of the few biodegradable polymers with mechanical properties that approach those of bone (the E-modulus of bone is 3 to 30 GPa) [14]. The ability to process PDLLA-based materials by stereolithography would allow to significantly advance the field of bone tissue engineering as optimised structures with regard to mechanical properties, cell seeding and culturing can then be prepared.

PDLLA networks can be formed by (photo-initiated) radical polymerisation of poly(lactide) oligomers end-functionalised with an unsaturated moiety such as a methacrylate-[15], acrylate-[16] or fumarate-[17] group. To be able to apply PDLLA macromers in stereolithography, the macromer must be in the liquid state. This can be achieved by heating or diluting. Reactive diluents such as methyl methacrylate, butane-dimethacrylate and N-vinyl-2-pyrrolidone have been used in regular photo-polymerisation reactions [15, 18] and in stereolithography [19, 20]. This, however, introduces significant amounts of a non-degradable component.

This work aims at developing a photo-curable PDLLA-based resin that is free of reactive diluents, and applying it in stereolithography. Ethyl lactate was employed as a non-

reactive diluent. This poses a challenge to the optimisation of the resins, as shrinkage upon extraction of the photo-polymerised networks can be quite significant. Methacrylate end-functionalised poly(D,L-lactide) oligomers of varying molecular architectures were synthesised and photo-crosslinked in the presence of ethyl lactate. Suitable resin compositions were used in stereolithography to prepare porous structures with pre-designed architectures at high resolution. Cell attachment and proliferation on PDLLA networks prepared by stereolithography were assessed.

Materials and Methods

Materials

D,L-lactide was obtained from Purac Biochem, The Netherlands. Hexanediol, glycerol, stannous octoate, methacryloyl chloride (MACI), hydroquinone, vitamin E, sodium pyruvate, N-methyl dibenzopyrazine methyl sulfate (PMS) and HistoChoice tissue fixative were purchased from Sigma-Aldrich, USA and used without further purification. Sorbitol, triethyl amine (TEA), eosin-hematoxylin solution (Fluka, Switzerland), ethyl lactate (Merck, Germany), and technical grade isopropanol and acetone (Biosolve, The Netherlands) were used as received. Irgacure 2959 (2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone) and Orasol Orange G were gifts from Ciba Specialty Chemicals, Switzerland. Lucirin TPO-L (ethyl-2,4,6-trimethylbenzoylphenylphosphinate) was a gift from BASF, Germany. Analytical grade dichloromethane (Biosolve, The Netherlands) was distilled from calcium hydride (Acros Organics, Belgium).

Foetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin were obtained from Lonza, Belgium. Alpha-Modified Eagle Medium (α MEM) and RPMI 1640 medium without phenol red were bought from Gibco, USA. Sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) was obtained from PolySciences, USA.

Polymer syntheses

Star-shaped oligomers were synthesised on a 30 g scale by ring opening polymerisation of D,L-lactide for 40 h at 130°C under an argon atmosphere, using stannous octoate as a catalyst. Hexanediol, glycerol and sorbitol were used as initiators, to prepare 2-armed, 3-armed and 6-armed oligomers, respectively. The molecular weights and arm lengths were varied by adjusting the monomer to initiator ratio. Proton-nuclear magnetic resonance spectroscopy (^1H -NMR, CDCl_3 , Varian 300 MHz) was used to determine lactide conversion and oligomer molecular weights.

Oligomers were functionalised by reacting the terminal hydroxyl groups with methacryloyl chloride (MACl) in dry dichloromethane under an argon atmosphere. The formed HCl was scavenged with triethyl amine (TEA). An excess of 20-50 mol% MACl and TEA per hydroxyl end-group was used.

The macromer solutions were filtered and precipitated into cold isopropanol. The isolated macromers were then washed with water and freeze-dried. Macromers with molecular weights lower than 1 kg/mol are soluble in isopropanol. These macromers were purified by washing the dichloromethane solutions with a saturated aqueous sodium bicarbonate solution, drying with magnesium sulphate, filtrating and evaporating the solvent under reduced pressure. The yield was 70 to 90 %, depending on macromer molecular weight.

$^1\text{H-NMR}$ was used to determine the degrees of functionalisation of the macromers. Throughout this paper, all macromers are labelled as MAxB , in which A stands for the number of arms and B for the arm length. For example, M3x0.6k stands for a 3-armed methacrylated PDLA macromer with each arm having a molecular weight of 0.6 kg/mol. The corresponding non-functionalised oligomer is designated as O3x0.6k , and the network obtained after photo-polymerisation of the macromer is designated as N3x0.6k .

As a reference material, high-molecular weight poly(D,L-lactide) (HMW PDLA) was synthesised by ring opening polymerisation under similar conditions. The polymer was purified by precipitation from acetone into water, and vacuum dried. Molecular weights were determined using a Viscotek GPCmax gel permeation chromatography setup with Viscotek 302 Triple Detection Array and CHCl_3 as an eluent with a flow of 1 mL/min. The following values were obtained: $\overline{M}_w = 4.3 \times 10^5$ g/mol, $\overline{M}_n = 3.6 \times 10^5$ g/mol and $[\eta] = 5.5$ dL/g.

Resin formulation and network preparation

To formulate liquid polymerisable poly(D,L-lactide) resins, the different macromers were diluted with ethyl lactate. The resin viscosity was determined over a range of diluent concentrations at 25 °C using a Brookfield DV-E rotating spindle viscometer, equipped with a small sample adapter. The shear rate was varied between 0.56 and 56 s^{-1} (Brookfield s21 spindle, rotating at 0.6 to 60 rpm). To prevent premature crosslinking 0.2 wt% hydroquinone was added to the liquid.

To obtain networks, the resins containing 2.0 wt% Irgacure 2959 as a biocompatible UV photo-initiator [21, 22], were irradiated with 365 nm UV-light for 15 min (Ultralum

crosslinking cabinet, intensity 3-4 mW/cm²). Silicone rubber moulds, covered with fluorinated ethylene-propylene (FEP) films to avoid oxygen inhibition, were employed to prepare specimens measuring 45x30x1.5 mm³.

Network characterisation

The obtained PDLLA networks were extracted with 3:1 mixtures of isopropanol and acetone, and dried at 90°C under a nitrogen flow for 2 d. Gel contents were determined in duplicate from the mass of the dry network after extraction (m_{dry}) and the macromer mass initially present in the resin (m_0). The specimens were then swollen in ethyl lactate for 2 d. The volume degrees of swelling of the networks were calculated using the swollen mass (m_{wet}) and the densities of PDLLA (1.25 g/mL) and ethyl lactate (1.03 g/mL):

$$Q = 1 + \frac{m_{wet} - m_0}{m_0} \times \frac{\rho_{PDLLA}}{\rho_{EL}}$$

Mechanical properties were determined in 5-fold in 3-point bending tests and in tensile tests using a Zwick Z020 universal tensile tester. The dimensions of the extracted and dried PDLLA networks for the bending tests were approximately 30x20x1 mm³. According to the ISO 178 norm used, the span-width and strain rate were adjusted to the specimen thickness. For the tensile tests, dumbbell-shaped samples were used according to the ISO 37-2 norm.

Water uptake of the different networks was assessed by conditioning extracted and dried samples in demineralised water at 37 °C for 15 hrs. Water uptake was defined as the relative increase in weight.

Stereolithography

To fabricate (porous) PDLA structures by stereolithography, a resin comprising 75 wt% M2x0.6k PDLA macromer, 19 wt% ethyl lactate, 6 wt% Lucirin TPO-L visible light photo-initiator, 0.025 wt% hydroquinone inhibitor and 0.2 wt% Orasol Orange G dye was formulated.

Tensile test specimens (ISO 37-2), films measuring 70x24x0.5 mm³ and a scaffold with a gyroid architecture were designed using Rhinoceros 3D (McNeel Europe) and K3DSurf (freeware obtainable from <http://k3dsurf.sourceforge.net>) computer software. The designs were built using an EnvisionTec Perfactory Mini Multilens stereolithography apparatus. This stereolithography apparatus (SLA) is equipped with a digital micro-mirror device [23] which enables projections of 1280 x 1024 pixels, each measuring 32x32 µm². Using a build platform step height of 25 µm, layers of resin were sequentially photo-crosslinked by exposure to a blue light pattern for 40 s. The intensity of the light was 20 mW/cm² and the wavelength ranged from 400 to 550 nm, with a peak at 440 nm.

After building, the scaffolds were extracted with 3:1 isopropanol and acetone mixtures and dried at 90 °C for 2 d. Imaging of gold-sputtered scaffolds was performed by scanning electron microscopy (SEM) employing a Philips XL30 FEG device with a 5.0 kV electron beam. Structural analysis was performed using micro-computed tomography (µCT) scanning on a GE eXplore Locus SP scanner at 6.7 µm resolution. The scan was carried out at a voltage of 80 kV, a current of 80 µA and an exposure time of 3000 ms. No filter was applied.

Cell culturing

Disk-shaped specimens (diameter 15 mm) of HMW PDLLA were punched out from compression moulded (140 °C, 250 kN) films. PDLLA network (N2x0.6k) disks were punched out from the films prepared by stereolithography. Prior to seeding, the samples were disinfected in 70 % isopropanol for 5 min, rinsed 3 times in phosphate-buffered saline (PBS) and incubated overnight in medium. Mouse pre-osteoblasts (MC3T3 cell line) were cultured in α MEM supplemented with 10 % FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 2 % PennStrep (penicillin-streptomycin). The cells were detached from the culture flask using 0.10 % (w/v) trypsin / 0.050 % (w/v) EDTA, after which 12×10^3 cells in 2 mL medium were pipetted onto each disk, in a 24-wells plate. For the TCPS controls, empty wells were used. The cells were cultured at 37 °C and 5 % CO₂ for 11 d, refreshing the medium at day 4 and day 8.

At each time point, specimens were fixed for 20 min in HistoChoice fixative solution and stained with eosin-hematoxylin. Light microscopy was used to visualise the adhered cells. Using NIH ImageJ software, the average area per cell on N2x0.6k PDLLA network films and TCPS was determined. Three images, each containing 25-80 cells, were evaluated. The HMW PDLLA films showed too many background irregularities to be analysed.

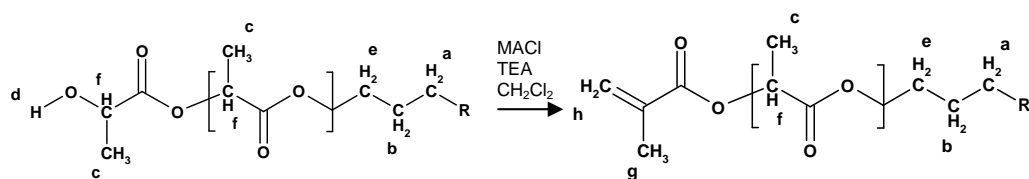
To quantify the number of live cells, other specimens were incubated for 60 min with colourless RPMI 1640 medium containing 1 mg/mL XTT and 7.66 μ g/mL PMS. After incubation, the relative absorbance of the supernatant at 450 nm (with reference to the absorbance at 620 nm) was measured in triplicate. Aliquots of 150 μ L were placed in a 96-well plate, and analysed using an SLT 340 ATTC plate-reader.

Mouse pre-osteoblasts were also seeded into the porous N2x0.6k PDLA scaffolds (5x5x5 mm³) with gyroid architecture prepared by stereolithography, by pipetting cell suspensions (600x10³ cells in 200 µL medium) onto each scaffold. The seeded structures were observed microscopically after 1 d as described for the films.

Results and Discussion

Polymers and macromers

The monomer conversions and the arm lengths of the synthesised lactide oligomers could be derived from ¹H-NMR spectra. A typical spectrum is depicted in figure 1. The lactide conversion was determined from the ratio of the peak areas corresponding to the monomer -CHCOO- protons (5.05 ppm) and the oligomer -CHCOO- protons (f, 5.15 ppm). In all cases, conversions reached were above 98 %. The degree of polymerisation *n* was determined from the ratio of the peak areas corresponding to the -CHCOO- end group protons (f', 4.35 ppm) and the -CHCOO- protons in the repeat unit (f, 5.15 ppm). Using *n*, the arm length (expressed as the number-average molecular weight per arm) was calculated.



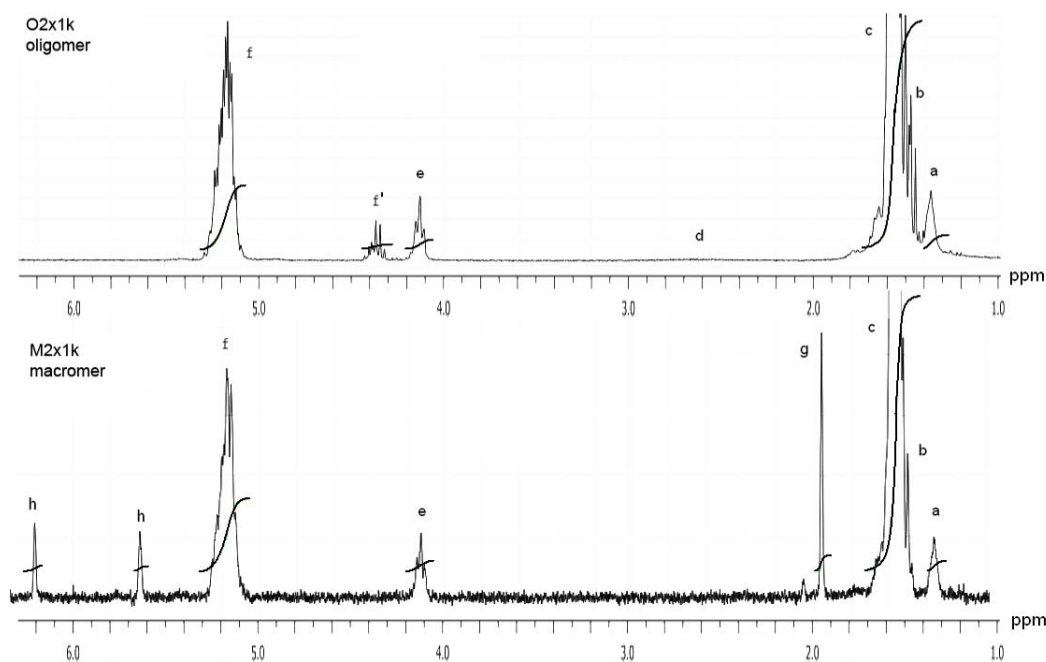


Figure 1: ^1H -NMR spectra of an O2x1k lactide oligomer (upper spectrum) and the corresponding M2x1k macromer (lower spectrum) after functionalisation with methacryloyl chloride. In the chemical structure, only one arm of the molecule is depicted.

The degrees of functionalisation of the obtained macromers were also determined by ^1H -NMR analysis; an overview is presented in table 1. The degree of functionalisation of the 2-armed macromers was determined from the peak areas corresponding to the methacrylate protons (h, 5.65 and 6.2 ppm) and the hexanediol $-\text{CH}_2\text{O}-$ protons (e, 4.1 ppm). The degrees of functionalisation of the 3-armed macromers were determined in a similar way, using the glycerol-residue peak (4.2 ppm). The peak corresponding to the sorbitol part of the 6-armed macromers is masked by the PDLLA $-\text{CHCOO}-$ (f) peak. Therefore, in case of these macromers, a degree of functionalisation of 100 % was inferred from the absence of $-\text{CHCOO}-$ end group peaks (f', 4.35 ppm).

Table 1: Average arm lengths and degrees of functionalisation (DF) of lactide macromers as determined by NMR analysis.

number of arms per macromer designated arm length	2		3		6	
	\bar{M}_n per arm (kg/mol)	DF (%)	\bar{M}_n per arm (kg/mol)	DF (%)	\bar{M}_n per arm (kg/mol)	DF (%)
0.2k	not prepared		0.22	100	not prepared	
0.6k	0.73	94	0.61	99	0.67	100
1k	1.1	100	1.1	97	1.0	100
5k	6.0	100	5.4	91	5.7	100

The synthesised macromers are termed M_Ax_B, in which A stands for the number of arms per molecule and B for the designated arm length

For use in stereolithography, a liquid resin that solidifies upon photo-polymerisation is required. In general, the viscosity of an oligomer or polymer depends on its molecular weight and architecture. When viscosities are too high to allow processing at a given temperature, for example when macromer molecular weights are high, reactive diluents are often used. However, in applications where degradation is desired, non-reactive diluents are preferred. In this case, issues of shrinkage and fragility of the structures during building need to be taken into consideration. For our purposes, ethyl lactate (EL) was found to be a suitable diluent. It is a relatively good solvent for the PDLA macromers, has a high boiling point of 151 °C and is not very volatile. It is also miscible

with acetone, isopropanol and water, allowing its facile removal from photo-polymerised structures.

The viscosities of the resins currently applied in stereolithography range from 0.25 Pa·s for a pentaerythritol tetra-acrylate resin to 5 Pa·s for ceramic suspensions [24]; a viscosity of approximately 1 Pa·s will be appropriate for our purposes.

The different macromers were diluted with varying amounts of EL, and their viscosities were determined at 25 °C. Over the measured range of shear rates, which was varied between 0.56 and 56 s⁻¹, the behaviour of the resins was essentially Newtonian. Figure 2 depicts the viscosities of different dilutions of the macromers at a shear rate of 9.3 s⁻¹. Clearly, the viscosities of all macromer formulations are strongly dependent on the amount of diluent.

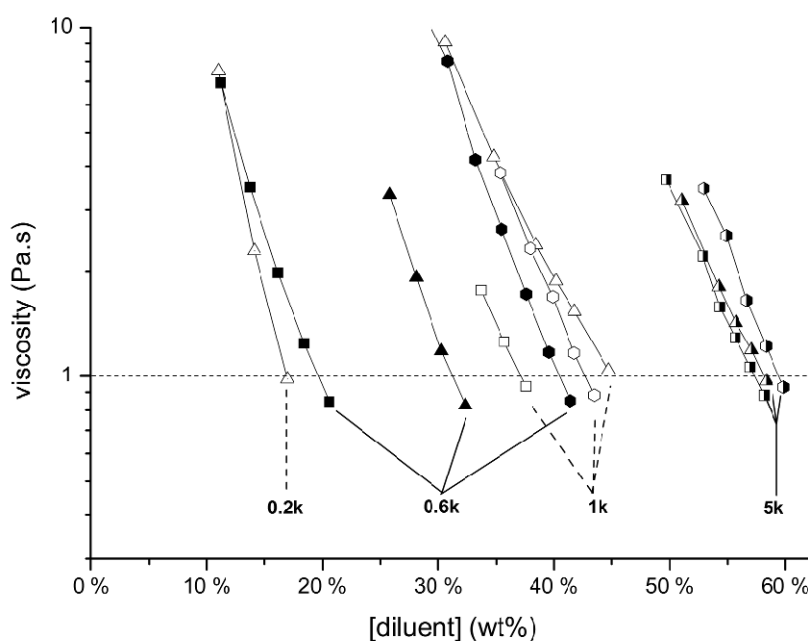


Figure 2: The resin viscosity of PDLLA macromers of different architectures and molecular weights as a function of the ethyl lactate diluent concentration.

Macromers with 2, 3 and 6 arms are represented in the figure by squares, triangles and hexagons, respectively. A viscosity of 1 Pa·s is appropriate for application in stereolithography.

The overall molecular weight of the macromers is determined by their arm lengths (the molecular weight per arm) and their molecular architecture (the number of arms per molecule). When comparing different macromers with a same number of arms, it can be seen that the overall molecular weight indeed has a significant effect on viscosity. Here, the length of the arms of the macromer primarily determines the amount of diluent that is needed to reach a suitable resin viscosity. Noticeably, for macromers of which the arm length is relatively high (5k), the number of arms has only a limited effect on the required amount of diluent. For macromers with relatively short arms (0.6k), the number of arms per molecule does have a considerable influence on viscosity. This implies that for these macromers, the overall molecular weight is the determining factor.

To obtain resins with a viscosity of 1 Pa·s, the different macromers were diluted with ethyl lactate. The required amounts, which will be referred to later, are given in table 2. As a photo-initiator Irgacure 2959 was added, and the resins were cast and photo-crosslinked in a UV cabinet.

Table 2: The ethyl lactate diluent concentration (in wt% of the resin) required to formulate PDLLA macromer resins with a viscosity of 1 Pa·s. PDLLA macromers with different architectures (See table 1 and figure 2) are compared.

number of arms per macromer designated arm length	2	3	6
0.2k	not prepared	16.7	not prepared
0.6k	19.1	32.7	40.5
1k	37.1	44.5	42.2
5k	57.3	58.1	59.4

Network characterisation

Networks were formed by photo-crosslinking different ethyl lactate-containing PDLLA macromer resins with viscosities of 1 Pa·s. All PDLLA networks had very high gel contents of 96 ± 3 %.

During the formation of a network, the glass transition temperature (T_g) increases as end-groups react and macromers are incorporated into the network. This can lead to vitrification, especially for high- T_g polymers. As the T_g of the network approaches the prevailing temperature, the forming network becomes glassy and crosslinking reactions are quenched [25]. The high gel contents of our PDLLA networks are the result of the presence of the non-reactive diluent. It acts as a plasticizer that increases the mobility of macromers and non-reacted end-groups. This enables the crosslinking reaction to proceed to high conversions and high gel contents.

As a consequence, networks with high T_g 's that exceed the photo-polymerisation temperature, can be obtained after removal of the non-reactive diluent. Figure 3 shows the T_g values of the PDLLA macromers and of the corresponding photo-crosslinked networks. The T_g of the macromers with different molecular architectures increases with increasing arm length. After the photo-crosslinking reaction, however, the T_g of the extracted networks increases with decreasing arm length of the macromers used. This is the result of an increasing crosslink density.

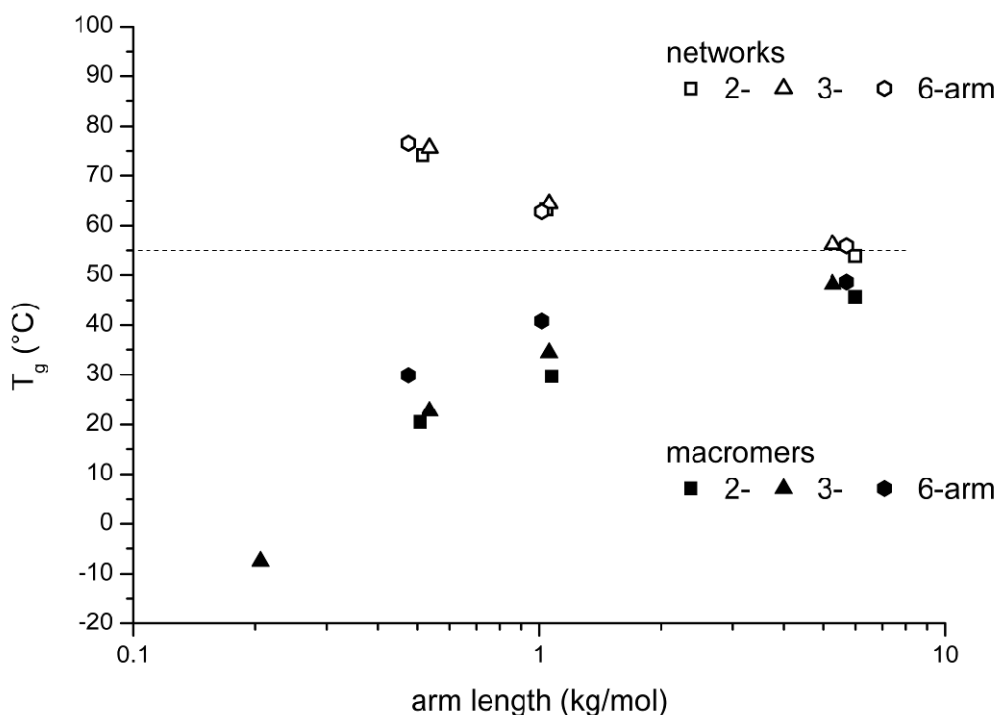


Figure 3: The glass transition temperatures (T_g) of PDLLA macromers of different architectures, and of the resultant extracted photo-crosslinked networks. The T_g of HMW PDLLA (55 °C) is indicated for comparison.

Compared to HMW PDLA, with a T_g of approximately 55 °C, PDLA networks with much higher glass transition temperatures can be obtained. For networks prepared from macromers with arm lengths of 0.6 kg/mol, T_g values close to 76 °C were determined. By DSC, a glass transition temperature could not be detected for the most densely crosslinked network (N3x0.2k). In contrast to the considerable effect of macromer chain length on the glass transition temperature of the networks formed, the effect of macromer architecture (the number of arms) is minimal.

The crosslink density of the PDLA networks formed, will also have an effect on their swelling behaviour in solvents. Equilibrium swelling experiments were performed in ethyl lactate, which is the non-reactive diluent in our stereolithography resins. The degree of swelling of the different extracted (and dried) PDLA networks is depicted in figure 4. Networks formed from macromers with arm lengths less than 1 kg/mol take up small amounts of EL. Networks formed from macromers with arm lengths of approximately 1 kg/mol swell to up to 1.4 times their dry volume, while networks formed from macromers with arm lengths close to 5 kg/mol swell up to nearly 6 times their dry volume.

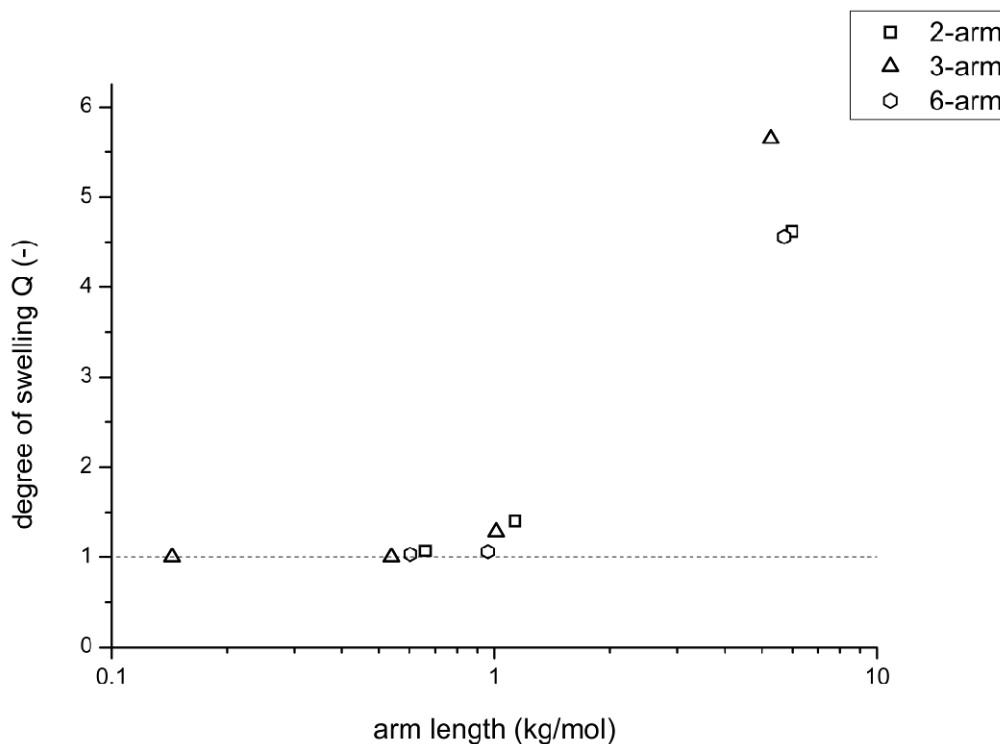


Figure 4: The degree of swelling in ethyl lactate of extracted PDLLA networks prepared from macromers with different architectures, as a function of the macromer arm length. The networks were prepared by photo-crosslinking PDLLA macromer resins diluted with ethyl lactate to a viscosity of 1 Pa·s, according to table 2. The measurements were performed in duplicate with an error of less than 4 %.

This is not only caused by the difference in molecular weights of the macromers used in the preparation of the networks, but also by the different amounts of diluent in the resins that are required to reach suitable viscosities for processing by stereolithography. For example, the resins based on macromers with 5 kg/mol arm lengths contain 57 to 60 wt% diluent, while the resin based on a 2-armed macromer with arm length of 0.6k contains

only 19 wt% of diluent, see table 2. At high dilutions of the resin, the density of trapped entanglements in the corresponding network is decreased. And additionally, the number of intra-molecular reactions is relatively high, leading to elastically inactive chains in the network [26]. Consequently, the effective crosslink density is lower when the photopolymerisations are carried out at high macromer dilutions. This results in higher degrees of swelling in ethyl lactate.

Mechanical properties

High-molecular weight poly(D,L-lactide) is a rigid, amorphous and glassy polymer. Its mechanical properties are appropriate for bone fixation devices or application in bone tissue engineering. Photo-crosslinked materials are generally based on low-molecular weight macromers, resulting in densely crosslinked networks that are often brittle. It can be expected that the mechanical behaviour of photo-crosslinked PDLA networks improves with increasing arm length of the precursors, approaching that of HMW PDLA.

The mechanical properties of the extracted and dried networks were determined in 3-point bending tests; the results are listed in table 3. An effect of the molecular architecture of the macromer on the properties of the networks cannot be observed. The mechanical properties of the networks are very similar to those of HMW PDLA. Only the most densely crosslinked network, N3x0.2k, exhibits significantly lower strains at failure. These brittle materials may be less suited for application in load-bearing constructs. The data show that to obtain PDLA networks with the most favourable mechanical properties, the arm length of the used macromer should not be lower than 0.6 kg/mol.

Table 3: The flexural properties of photo-crosslinked PDLLA networks and HMW PDLLA. Determinations of the flexural modulus (E_f), flexural strength (σ_f) and strain at failure (ϵ) were performed in 5-fold; the results are presented as average values \pm standard deviations.

number of arms per macromer designated arm length	2	3	6
0.2k	not prepared	$E_f = 3.6 \pm 0.4$ GPa $\sigma_f = 79 \pm 19$ MPa $\epsilon = 2.4 \pm 0.5$ %	not prepared
0.6k	$E_f = 2.9 \pm 0.1$ GPa $\sigma_f = 94 \pm 1$ MPa $\epsilon = 5.0 \pm 0.1$ %	$E_f = 2.5 \pm 0.6$ GPa $\sigma_f = 83 \pm 22$ MPa $\epsilon = 6.2 \pm 0.2$ %	$E_f = 2.5 \pm 0.5$ GPa $\sigma_f = 93 \pm 14$ MPa $\epsilon = 6.1 \pm 0.2$ %
1k	$E_f = 2.5 \pm 0.5$ GPa $\sigma_f = 80 \pm 8$ MPa $\epsilon = 5.3 \pm 0.5$ %	$E_f = 3.5 \pm 0.2$ GPa $\sigma_f = 118 \pm 16$ MPa $\epsilon = 4.5 \pm 1.1$ %	$E_f = 2.7 \pm 0.3$ GPa $\sigma_f = 92 \pm 1$ MPa $\epsilon = 5.6 \pm 1.0$ %
5k	$E_f = 3.4 \pm 0.1$ GPa $\sigma_f = 89 \pm 8$ MPa $\epsilon = 4.2 \pm 0.8$ %	$E_f = 2.9 \pm 0.1$ GPa $\sigma_f = 87 \pm 2$ MPa $\epsilon = 4.9 \pm 0.6$ %	$E_f = 3.4 \pm 0.4$ GPa $\sigma_f = 97 \pm 6$ MPa $\epsilon = 4.1 \pm 0.3$ %
HMW PDLLA	$E_f = 2.9 \pm 0.6$ GPa $\sigma_f = 88 \pm 14$ MPa $\epsilon = 4.9 \pm 1.1$ %		

Stereolithography

While rapid prototyping methods such as fused deposition modelling and selective laser sintering are restricted to simple architectures, stereolithography allows the preparation of structures with more advanced designs. To prepare PDLLA structures by stereolithography, a resin based on the M2x0.6k macromer was formulated. This macromer requires only 19 wt% of non-reactive ethyl lactate diluent to reach the appropriate resin viscosity. This will result in minimal shrinkage upon extraction and drying of a built structure. Moreover, the resulting network has excellent mechanical properties.

Stereolithography is a layer-by-layer fabrication method [27]. Complex structures can be built by illuminating sequential layers of a polymerisable resin using digital pixel masks or arrays of mirrors. In stereolithography, the thickness of a solidified layer (cure depth, C_d in μm) is controlled by the light irradiation dose E (mJ/cm^2). A plot of the cure depth versus the irradiation dose is termed a working curve [27] and can be described by:

$$C_d = D_p \ln \frac{E}{E_c}$$

In a specific SLA setup, curing of the resin is characterised by a critical energy E_c (mJ/cm^2) and a penetration depth D_p (μm). E_c is the minimum energy required to reach the gel-point and start forming a solidified layer. The penetration of light into the resin is characterised by D_p .

To achieve good attachment between layers, the conversion of the macromer at the interface of the layers should be slightly higher than the gel point. In our experiments, a light irradiation dose that is 10 % higher than that required to obtain a cure depth that

equals the step height of the build platform, was found to be well suited. This overexposure results in (further) curing into the preceding layer. Pixels or volume elements which according to the design were to remain uncured, will now have partially polymerised. This inevitable overcure will affect pore size and geometry when building porous structures. A high value of the extinction coefficient of the resin corresponds to a small D_p , and will allow minimal overcure and most accurate control of the building process. This is due to the fact that conversion of the photo-polymerisation reaction versus the distance from the resin surface is an exponentially decaying function (according to Beer-Lambert).

Figure 5 shows working curves for resins prepared from the M2x0.6k PDLLA macromer, which further contained 19 wt% ethyl lactate and 6 wt% photo-initiator. From the slopes of the working curves, it can be seen that the penetration depth of the resin decreased from 301 μm to 78 μm upon addition of 0.2 wt% Orasol Orange G dye. Under our experimental building conditions, this will reduce the overcure into a preceding layer from approximately 29 μm to only 7 μm .

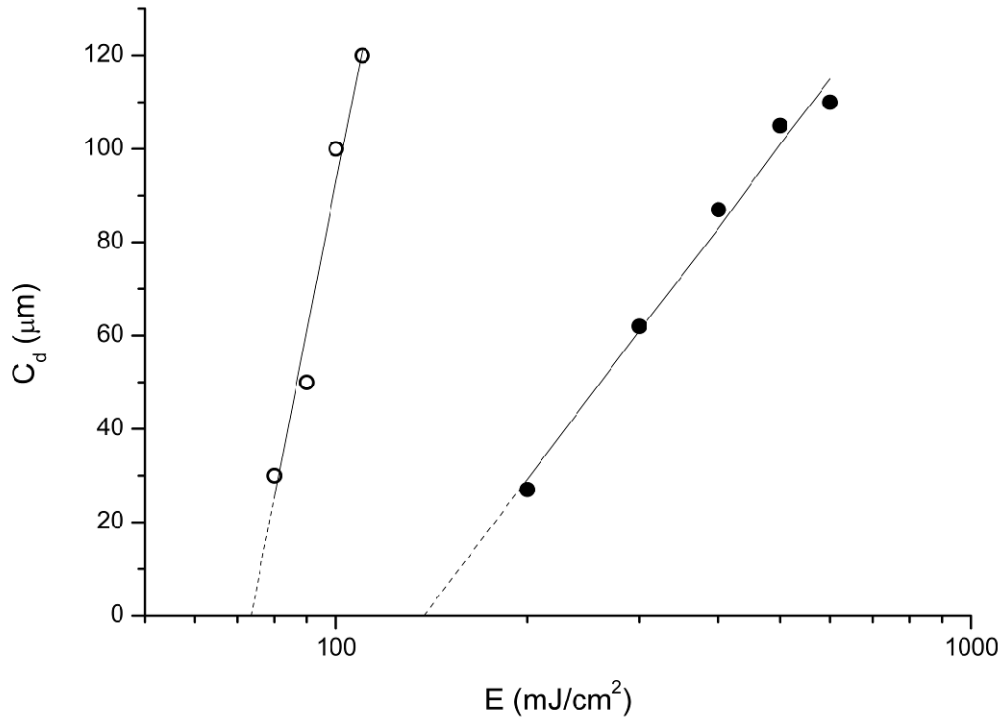


Figure 5: Stereolithography working curves of M2x0.6k PDLLA macromer-based resins containing 0.2 wt% Orasol Orange G dye (●) and containing no dye (○).

With this dye-containing resin, stereolithography was used to build ISO 37-2 non-porous, dumbbell-shaped specimens which were then subjected to tensile testing. The tensile strength, Young's modulus and elongation at break of the N2x0.6k PDLLA network photo-crosslinked in the SLA were 56 ± 2 MPa, 3.3 ± 0.2 GPa and 1.9 ± 0.1 %, respectively. These values were very reproducible and compared favourably with those of compression moulded HMW PDLLA specimens. The latter values were respectively 44 ± 5 MPa, 2.7 ± 0.1 GPa and 2.0 ± 0.5 %.

Cell culturing

To demonstrate the suitability of these materials for use in medical applications such as bone tissue engineering, mouse pre-osteoblasts (from the MC3T3 cell line) were seeded and cultured on photo-crosslinked PDLLA disks. These disks were punched out from a film that was also prepared from the M2x0.6k macromer-based resin by SLA. Compression-moulded HMW PDLLA was used as a reference material, and tissue culture polystyrene (TCPS) as a positive control. Figure 6 presents light microscopy images of the cells on the materials at 1 d, 4 d and 11 d after seeding. The images clearly show an increase in cell density in time. (The layering which is visible in the images of the N2x0.6k PDLLA networks results from the layer-by-layer nature of the stereolithography process.) Figure 7 shows high-magnification images of the pre-osteoblasts. The cells show similar spread morphologies on all materials. The cells had an average area of $3.9 \times 10^2 \mu\text{m}^2/\text{cell}$ for the N2x0.6k PDLLA networks and $5.4 \times 10^2 \mu\text{m}^2/\text{cell}$ for TCPS.

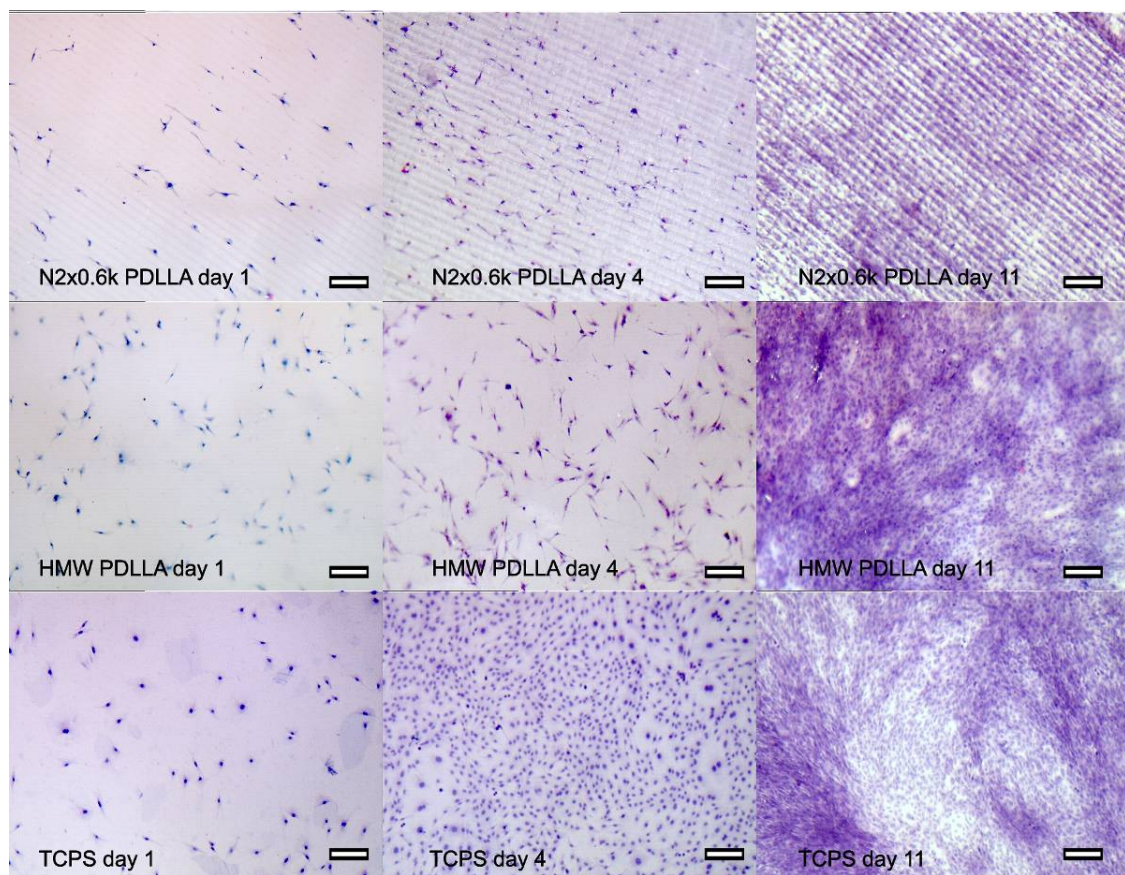


Figure 6: Light microscopy images of mouse pre-osteoblasts cultured on PDLLA network (N2x0.6k) films vertically prepared by stereolithography. HMW PDLLA and TCPS were used as controls. The scale bars represent 200 µm.

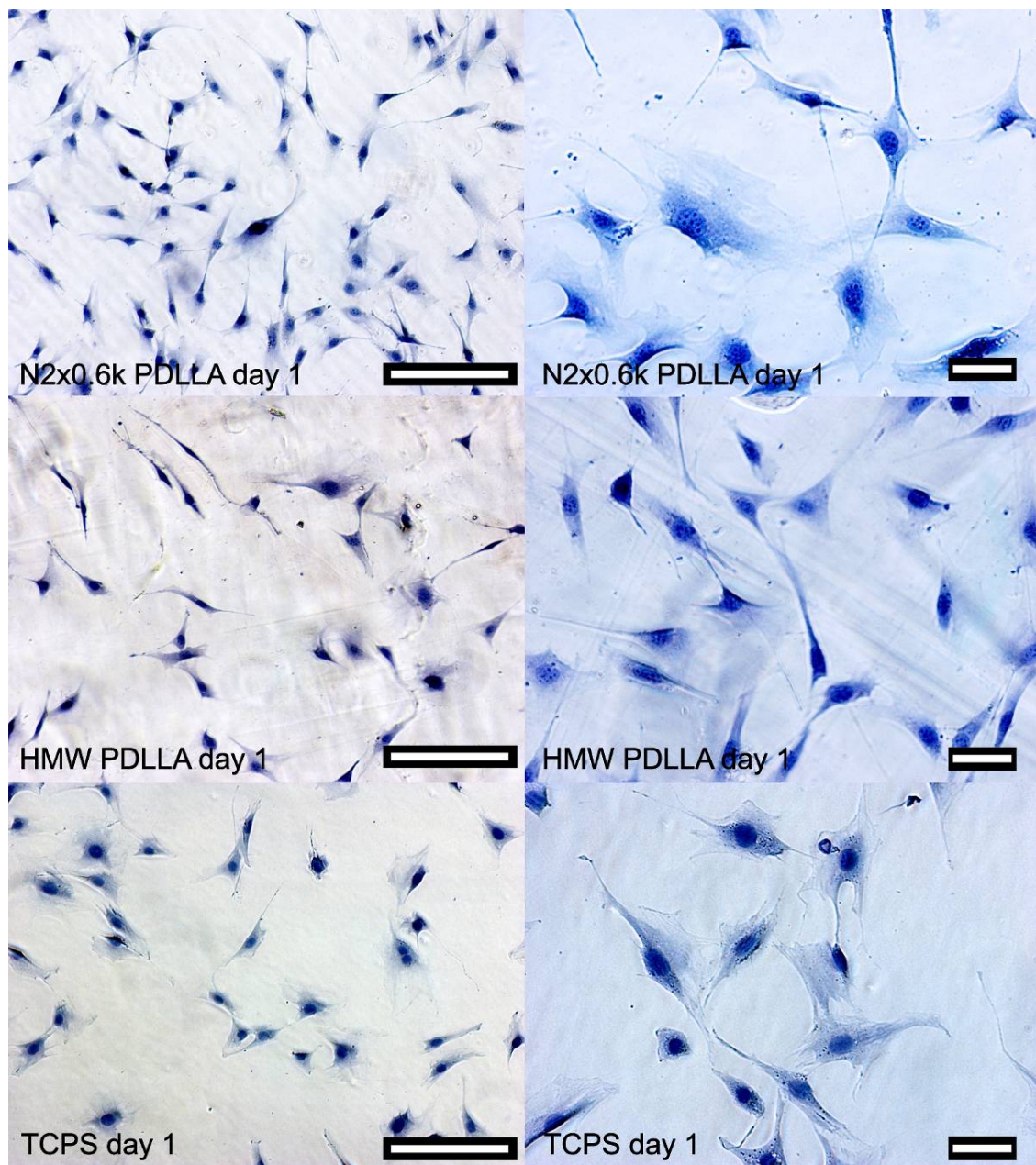


Figure 7: Light microscopy images showing the spreading of mouse pre-osteoblasts after 1 day of culturing on PDLLA network (N2x0.6k) films horizontally prepared by stereolithography. HMW PDLLA and TCPS were used as controls. The scale bars represent 100 μm.

In figure 8, the cell numbers determined by the XTT assay are given as a function of time. For all materials, confluency (4.5×10^5 cells/cm²) was reached within 11 d. Although initially the number of adhering cells on TCPS was higher than on HMW PDLLA and PDLLA network films prepared by stereolithography, the proliferation rates after day 1 are comparable. The doubling time is approximately 1 d. It can be stated that N2x0.6k PDLLA network films obtained by stereolithography are excellent substrates for the culturing of cells.

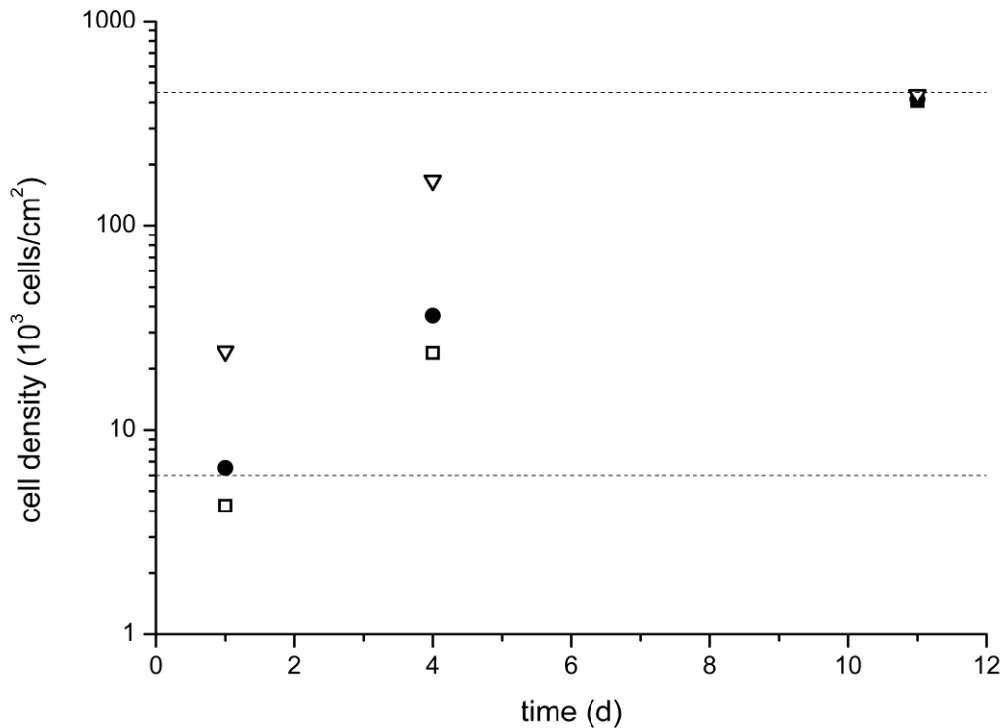


Figure 8: Proliferation of mouse pre-osteoblasts on PDLLA network films prepared from M2x0.6k macromers by stereolithography (●), on HMW PDLLA (□) and on TCPS (▽). The initial seeding density was 6×10^3 cells/cm², the cell density at confluency was 450×10^3 cells/cm².

Tissue engineering scaffolds

The ability to prepare PDLLA networks with excellent mechanical properties and cell culturing characteristics, now allows the preparation by stereolithography of porous scaffolds for tissue engineering. Under our experimental conditions, it is possible to build relatively large structures (up to 42x33x200 mm³ in size) at high resolutions. The size of the smallest features that can be built are determined by the size of the light pixels (32x32 μm² in the x and y direction), the layer thickness (25 μm) and the overcure (only 7 μm for our M2x0.6k PDLLA macromer-based resin).

We prepared porous PDLLA network scaffolds with a gyroid architecture. This gyroid architecture is mathematically defined, allowing precise control of porosity and pore size of a fully interconnected pore network [28]. Such built structures are presented in figure 9. Under the applied experimental conditions, building times were approximately 5 h. Our SLA setup allows the simultaneous building of up to 30 such structures.

From the μCT data, the structural parameters of the built scaffolds after removal of the diluent were determined [19, 29]. It was found that in all directions the shrinkage was 10 %. As the shrinkage is isotropic, the designed architecture is preserved and porosity is almost unaffected; in the design the porosity was 75 %, while a porosity of 73 % was determined. This shrinkage occurs upon removal of the diluent, and can be accounted for in designing structures. μCT data also revealed a narrow pore size distribution; 83 % of the pore volume was occupied by pores ranging in size from 170 to 240 μm, with an average pore size of 200 μm. The specific surface area was 47 mm²/mm³.

Cell seeding of porous structures prepared from hydrophobic polymers, such as PDLLA is difficult [30]. All dry photo-crosslinked PDLLA networks absorbed only 0.6 ± 0.2 wt%

of water. For HMW PDLLA, a slightly higher water uptake of 0.9 ± 0.1 wt% was determined. In scaffolds prepared by salt-leaching or freeze-drying methods, the penetration of a cell suspension is further hindered by the high tortuosity and poor interconnectivity of these pore networks.

The very open structure of the gyroid architecture facilitates the penetration of water into our PDLLA scaffolds prepared by stereolithography. The cell seeding could be performed effortlessly by simply pipetting the cell suspension onto the scaffold. Figure 9D shows a cross-section of such a scaffold 1 d after seeding with mouse pre-osteoblasts. It is clear that the cells are well attached and homogeneously distributed throughout the porous scaffold.

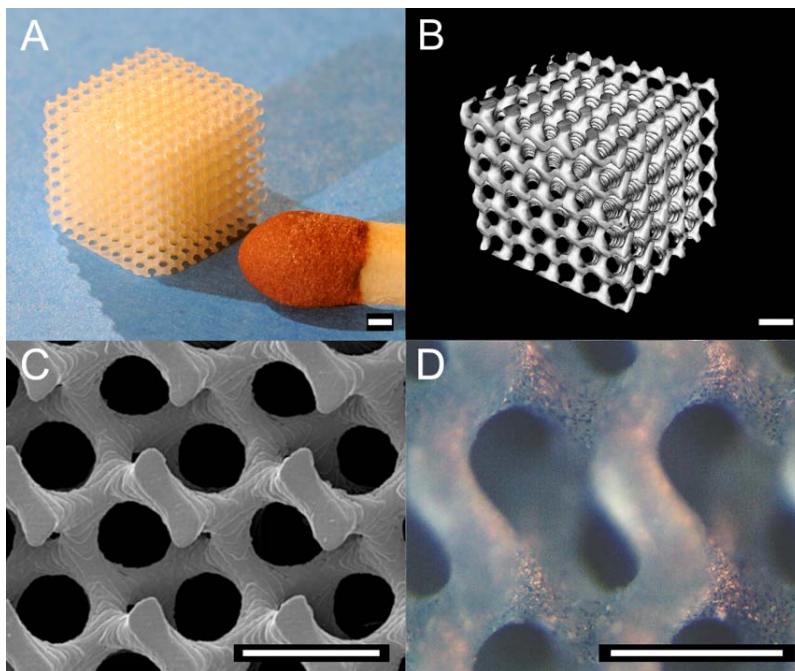


Figure 9: Images of PDLLA network scaffolds with a gyroid architecture, built by stereolithography. (A) photograph, (B) μ CT visualisation and (C) SEM image. In (D) a light microscopy image is shown of a scaffold seeded with mouse pre-osteoblasts after 1 d of culturing. Scale bars represent 500 μ m.

The excellent mechanical properties, the very good compatibility with cells, and the ability to build pre-designed (porous) architectures from a PDLLA macromer-based resin by stereolithography, makes these degradable materials very useful in medical applications. Currently, we are investigating the degradation behaviour of these photo-crosslinked PDLLA networks.

Conclusions

A resin based on poly(D,L-lactide) macromers and a non-reactive diluent was developed and applied in stereolithography. Designed solid structures and porous scaffolds were prepared and characterised. D,L-lactide oligomers with different molecular weights and molecular architectures were functionalised with methacrylate end-groups, and photo-polymerised in the presence of ethyl lactate as a non-reactive diluent. Networks with high gel contents were obtained. After removal of the diluent, materials with excellent mechanical properties, similar to those of linear high molecular weight PDLLA, were obtained. Films and porous scaffolds with gyroid architecture were prepared by stereolithography, using a liquid resin based on a 2-armed PDLLA macromer and ethyl lactate. Mouse pre-osteoblasts readily adhered and proliferated well on these networks. As a result of the open pore architecture, these hydrophobic scaffolds could be seeded without difficulty. It is anticipated that degradable structures prepared by stereolithography from these resins will be very well suited for use as bone tissue engineering scaffolds.

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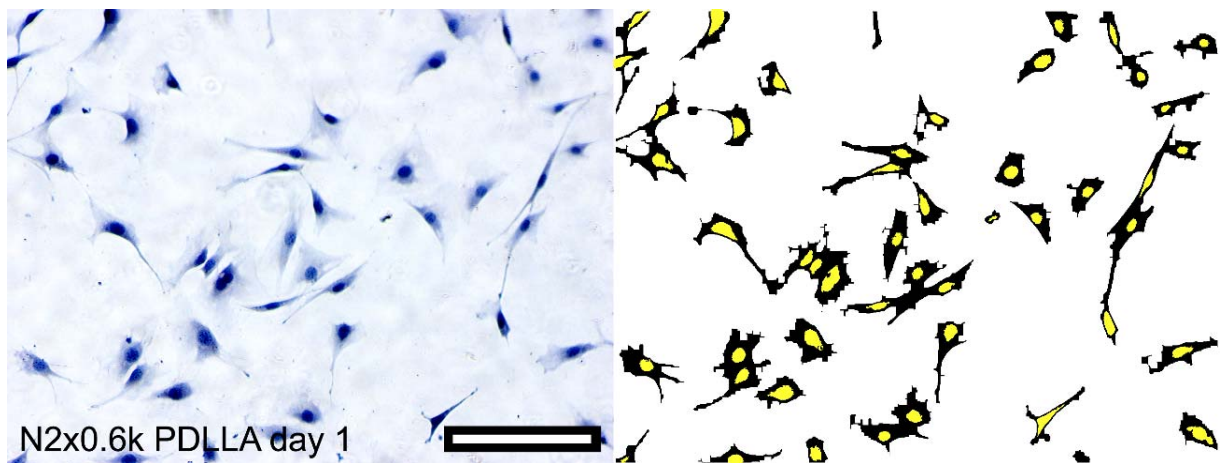
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Supporting Data



Left: Light microscopy image of mouse pre-osteoblasts cultured 1 day on a N2x0.6k PDLLA network prepared by stereolithography.

Right: The image after processing with ImageJ analysis software. The average cell area is determined as the total cell area (black + yellow) divided by the number of cell nuclei (yellow particles).